12-14-00 09/719658 Rec'd PCT/PTO 13 DEC 2000

FORM PTO-1390 (REV 10-2000)

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S APPLICATION NO (If known, see 37 CFR 1 5)
102180-101	09/719658

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB99/02157	6 July 1999	6 July 1998

TITLE OF INVENTION: VASCULOPROTECTOR

APPLICANT(S) FOR DO/EO/US: KARO BIO AB

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. [ ] The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Copy of International Search Report (PCT/ISA/210)
    PCT Request (Form PCT/RO/101)
    PCT Written Opinion (Form PCT/IPEA/408)
    International Preliminary Examination Report (Form PCT/IPEA/416)

I hereby certify that this Application Transmittal and documents referred to as being enclosed therein are being deposited with the United States Postal Service on this date December 13, 2000 in an envelope as "Express Mail Post Office to Addressee" Mailing Label No. EL516724527US to the Commissioner for Patents, Washington, D.C. 20231.

Todd E. Garabedian
Name of Person Mailing Paper

Todd E. Garabedian
Signature of Person Mailing Paper

JC20 Rec'd PCT/PTO 13 DEC 2000

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/719658	PCT/GB99/02157	102180-101

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	29 - 20 =	9	X **$18.00**	$ 162.00	
Independent claims	8 - 3 =	5	X **$80.00**	$ 400.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1,422.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$ -711.00	
SUBTOTAL =				$ 711.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ 0.00	
TOTAL NATIONAL FEE =				$ 711.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 0.00	
TOTAL FEES ENCLOSED =				$ 711.00	
				Amount to be refunded:	$
				charged:	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 23-1665 in the amount of $ 711.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-1665. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Docket Coordinator
Intellectual Property Section
WIGGIN & DANA
One Century Tower
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Todd E. Garabedian

SIGNATURE:

Todd E. Garabedian, Ph.D.

NAME

39,197

REGISTRATION NUMBER

Dec. 13, 2000

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application	:	Pekka Hayry et al.
Serial No.	:	
Filed	:	HEREWITH
For	:	VASCULOPROTECTOR
Examiner	:	
Attorney Docket	:	102180-100
Group Art Unit	:	

* *

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231 on _____.

By _____
Todd E. Garabedian, Ph.D.
Registration No. 39,197
Attorney for Applicants

* *

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to calculating the filing fee, please amend the above-identified patent application as follows:

IN THE SPECIFICATION:

At page 1, following the title, please insert:

--BACKGROUND OF THE INVENTION

Field of the Invention--.

At page 1, following line 5, please insert:

Brief Description of the Related Art --.

At page 3, following line 20, please insert:

--SUMMARY OF THE INVENTION--.

At page 7, following line 14, please insert:

--BRIEF DESCRIPTION OF THE DRAWINGS--.

At page 8, following line 5, please insert:

--DETAILED DESCRIPTION OF THE INVENTION--.

At page 17, before claim 1, please insert:

--WHAT IS CLAIMED IS:--

IN THE CLAIMS

In claim 4, line 1, please delete "or claim 2".

In claim 7, line 1, please delete "or 6".

In claim 18, line 1, please delete "any one of claims 13 to 17" and insert therefor --claim 13--.

In claim 20, line 1, please delete "any preceding claim" and insert therefor -claim 1--.

In claim 21, line 1, please delete "any one of claims 9 to 20" and insert therefor --claim 9--.

In claim 22, line 1, please delete "any one of claims 13 to 21" and insert therefor --claim 13--.

In claim 25, line 1, please delete ", 23 or 24".

In claim 29, line 1, please delete "or 28".

REMARKS

Claims 1-29 are presented for Examination.

Applicants herein amend the claims to remove multiple claim dependencies to reduce the filing fee. Applicants respectfully request that the filing fee be calculated on the basis of the above amended claims.

Applicants further amend the specification to add proper section headings in accordance with U.S. practice. No new matter is added herewith.

If the Examiner believes a telephone conference would aid in the continued prosecution of this application, the Examiner is invited and encouraged to contact Applicants' representative at the telephone number listed below.

Respectfully submitted,

PEKKA HAYRY ET AL.

By Todd E. Garabedian

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Date: 13 December 2000

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VASCULOPROTECTOR

This invention relates to the use of ligands of the second estrogen receptor, ERβ, or compounds which affect ERβ for vasculoprotection, that is to say in inducing protective effects in the vascular wall particularly in fibroproliferative disorders (such as atherosclerosis, ateriosclerosis, diabetic and autoimmune angiopathies), after injury (such as restenosis after angioplasty and bypass surgery) and in chronic allograft rejection.

Recently, a second estrogen receptor, ERβ, has been revealed (WO97/09348).

Vascular intimal dysplasia and remodelling are characteristic features of reinjury following balloon angioplasty, coronary bypass surgery (Holmes, D. *et al.* Am J Cardiol (1984); **53**: 77C-81C, Holmes, D. *et al.* J Am Coll Cardiol (1988); **22**: 1149-55), and in chronic allograft rejection (Lemström KB, Koskinen PK. Circulation (1997); **96**: 1240-1249, Häyry, P. *et al.* Immunol Rev (1993) Aug; **134**: 33-81, Häyry P. *et al.* Faseb J (1993); **7**: (11): 1055-60). The initial response to vascular injury is inflammatory and involves the attraction of lymphocytes, macrophages and thrombocytes to the site of injury and the secretion of cytokines, eikosanoids and growth factors (Ross. R. Nature (1993); **362**(6423): 801-9). Under the influence of growth factors and cytokines, smooth muscle cells (SMC) proliferate and migrate from the media into the intima and contribute to intimal hyperplasia and stenosis.

Estrogen has several protective effects on the vascular wall (Farhat MY *et al.* J Pharmacol Exp Ther (1996); **276**: 652-7). Some of these are rapid, presumably direct membrane effects, whereas others require transcriptional activation of genes (Farhat MY. *et al.* Biochem Pharmacol (1996); 51(5): 571-6). The inhibitory effect of estrogen on the replication, migration and extracellular matrix deposition by vascular smooth muscle cells, the key event in vascular fibroproliferative dysplasias, is presumably a genomic effect mediated through a variety of mechanisms including regulation of several growth factors and/or their receptors and possibly by a direct antiproliferative effect of estrogen on smooth muscle cells (Farhat MY, *et al.* Faseb J (1996); **10**(5): 615-24).

The vasculoprotective effect of estrogen was first demonstrated in population studies in humans, where estrogen replacement therapy demonstrated a protective effect on atherosclerotic vascular disease in post menopausal women (Stampfer M J *et al* (1991) *N. Engl. J. Med.* **325**: 756-762; Grady D *et al.* (1992) *Ann. Intern. Med.* **117**: 1016-1037), as later confirmed in monkeys (Wagner JD *et al.* Metabolism (1997); **46**(6): 698-705). Later, the vasculoprotective effect has been documented in more detail in animal models and *in vitro*. Estrogen has been found to inhibit the intimal thickening after mechanical carotid balloon injury in rabbits (Foegh ML *et al.* J Vasc Surg (1994); **19**(4): 722-6), rats (Chen SJ. *et al* Circulation (1996); **93**(3): 577-84) and in mice (Sullivan TJ *et al.* J Clin Invest (1995); **96**: 2482-8), as well as the immunologically-induced vascular fibroproliferative dysplasia in rabbit aorta (Cheng LP, *et al.* Transplantation (1991); **52**(6): 967-72) and heart (Foegh ML. *et al.* Transplant Proc (1987): 90-5) allografts. *In vitro*, it has been demonstrated that estrogen inhibits migration and replication of vascular smooth muscle cells (Akishita M *et al.* Atherosclerosis (1997); **130**(1-2): 1-10, Kolodgie FD, *et al.* Am J Pathol (1996); **148**(3): 969-76, Morey AK, *et al.* Endocrinology (1997); **138**(8): 3330-9, Suzuki A, *et al.* Cardiovasc Res (1996); **32**(3): 516-23). These observations are consistant with findings in reporter gene assays that functional estrogen receptors are expressed in vascular smooth muscle cells of bovine (Balica M. *et al.* Circulation (1997); **95**(7): 1954-60, rat ((Bayard F. *et al.* Endocrinology (1995); **136**(4): 1523-9, Bayard F. *et al.* Ciba Found Symp (1995); **191**(122): 122-32, Bei M *et al.* J Steriod Biochem Mol Biol (1996); **58**(1): 83-8), guinea pig (Bhalla RC, *et al.* Am J Physiol (1997): H1996-2003), and human (Karas RH, *et al.* Febs Lett (1995); **377**(2): 103-8) origin.

In addition to being anti-proliferative and anti-migratory to smooth muscle cells, estrogen may also display vasculoprotective effects via the vascular wall endothelium. Functional estrogen receptors have been demonstrated in endothelial cells (Venkov CD *et al.* Circulation (1996); **94**: 727-33). Estrogen downregulates cytokine-induced adhesion molecule expression in human endothelium *in vitro* (Caulin GT. *et al.* J Clin Invest (1996); **98**(1): 36-42), it is anti-apoptotic to endothelial cells (Spyridopoulos, I *et al.* Circulation (1997); **95**(6): 1505-14) and it enhances functional endothelial recovery after denudation assay *in vivo* (Krasinski K *et al.* Circulation (1997); **95**(7): 1768-72).

There are also additional, indirect pathways whereby estrogen may mediate vasculoprotective effects. In addition to being directly anti-proliferative to smooth muscle cells and protective to the vascular endothelial cells, the estrogen effect may be mediated indirectly via lipoprotein metabolism and via promoting vasodilation by stimulating prostacyclin and nitric oxide synthesis and via regulation of the cell membrane voltage-dependent calcium channels resulting in inhibition of extracellular calcium mobilization and flux (see Farhat MY. *et al.* J Pharmacol Exp Ther (1996); **276**(2): 652-7, Farhat MY. *et al.* Biochem Pharmacol (1996); **51**(5): 571-6). None of these mechanisms alone explain the beneficial effect of estrogen. For example, although the endothelial nitric oxide synthetase (e-NOS) mRNA and protein are upregulated in vascular endothelium by estrogen *in vitro* (MacRitchie AN, *et al.* Circ Res (1997); **81**(3): 355-62), the effect is only partially inhibited by the e-NOS inhibitor NAME (Holm P *et al.* J Clin Invest (1997); **100**(4): 821-8).

The development of vasculoprotective drug therapies based on the protective effect of estrogen has been difficult, as it has not been possible to differentiate the desired vasculoprotective effect of estrogen from its undesirable effects on the reproductive system - e.g. its uterotrophic effect.

Recent work on the vasculoprotective effect of estrogen in ERα-deficient mice by Iafrati and coworkers (Iafrati MD *et al.* Nat Med (1997); **3**(5): 545-8), suggests that ERα may not be responsible for the vasculoprotective estrogenic response.

The inventors have unexpectedly managed to differentiate the vasculoprotective effect of estrogen from its uterotrophic effect using ligands with different binding affinity to ERα and ERβ. The inventors have discovered that the genistein, an isoflavonic phytoestrogen, which shows approximately 20 x higher binding affinity to ERβ compared its binding affinity for ERα displays a full vasculoprotective effect but is devoid of any uterotrophic effect. In addition, the inventors have demonstrated that ERβ is strongly upregulated in the vascular wall as a consequence of injury, whereas ERα remains expressed at a constitutive background level only.

The discovery of a second estrogen receptor ERβ, and the recent finding that disruption of the "classical" ERα gene in mice preserves the vasculoprotective effect of estrogen, offer better targeting of estrogen in vasculoprotective drug therapies. The inventors have unexpectedly demonstrated that, after endothelial denudation injury of rat carotid artery, ERα mRNA (and protein) are constitutively expressed at a low level in the vascular wall, whereas the expression of ERβ mRNA increases >30-fold after injury. In *in situ* hybridization, the ERβ mRNA co-localizes with the replicating and migrating SMC in the media and in the neointima. Treatment of ovariectomized female rats on a soybean deficient diet with the isoflavonic phytoestrogen genistein, which shows approximately 20x higher binding affinity to ERβ than to ERα, and with 17β -estradiol, which does not differentiate between the two receptor subtypes, at a dose range of 0.00250 through 2.50 mg/kg/d, provides on both occasions a dose-dependent vasculoprotective effect. However, treatment with 17β-estradiol only, but not with genistein, is accompanied by a dose-dependent uterotrophic effect. Thus the vaculoprotective effect of estrogen has been for the first time differentiated from the uterotrophic effect using ligands with different binding affinity to ERβ vs ERα. The *in vivo* experiments used genistein doses of less than 2 mg/kg/d, which is well known below the dose level (50 - 100 mM) where the protein tyrosine kinase inhibitory effect of genistein and isoflavones have been demonstrated. As genistein at this dose range is likely to function via the ERβ, and is devoid of inhibitory effect on protein tyrosine kinases, the vasculoprotective effect of genistein is therefore mediated by ERβ.

The colocalization of ERβ mRNA in *in situ* hybridization on the replicating/migratory smooth muscle cells during the replicative and migratory bursts after endothelial trauma, is of relevance in explaining the results obtained. First, on day 7 after denudation injury the endothelial regrowth from both ends of the vessel is only at its very beginning (Clowes AW, *et al.* Lab Invest (1983); **49**(3): 327-33) and the dose dependent inhibitory effect with both ligands may be discussed in terms of inhibition of smooth muscle cell replication, only. Although the binding affinity of genistein to ER-β is only 20-fold better than 17β-estradiol, (the best pair of ligands at the moment to differentiate between the two receptors -Kuiper, G *et al* Endocrinology (1997); **138**(3): 863-70), the anti-proliferative

effect and the effect on intimal thickening vs. the uterotrophic effect were clearly different.

In regard to intimal thickening and *in vivo* replication after endothelial denudation, the linear plots show a slight advantage for genistein vs. 17-β-estradiol. This advantage is also seen in the *in vitro* vascular smooth muscle cell inhibition studies. However, at the tested dose range genistein displayed no uterogenic effect *in vivo*, whereas the uterogenic effect of 17-β-estradiol was clearly visible. Thus, it can be calculated that the vasculoprotective effect of the estrogen is most likely mediated via ERβ. This is clearly supported by the observation of Iafrati *et al* Nat Med (1997); **3**(5): 545-8 where selective disruption of the ERα gene in mice did not have an effect on the vasculoprotective effect of estrogen.

Therefore, in summary, the inventors have demonstrated that ERβ is strongly upregulated in the vascular wall as a consequence of injury, whereas ERα remains expressed at constitutive low background level, only. *In situ* hybridization demonstrated that ERβ mRNA co-localizes on the replicating and migrating vascular smooth muscle cells in the media and neointima suggesting that both genes are transcribed and expressed in functional form. This was tested by using two different ligands with approximately 20-fold affinity difference for ERα and ERβ, 17-β-estradiol and genistein. These two ligands clearly differentiated the vasculoprotective vs. uterogenic effect. Within the dose range tested both of the ligands demonstrated a dose-dependent vasculoprotective effect, whereas only 17-β-estradiol but not genistein demonstrated a dose-dependent uterotrophic effect. Finally, the anti-proliferative effect of these two ligands on vascular smooth muscle cell cultures deriving from rat aorta was confirmed *in vitro*: a dose-dependent inhibition of smooth muscle cell proliferation was again observed.

Taken together, the results presented in this study and previous observations strongly suggest that the vascular protective effect of estrogen is mediated predominantly or exclusively by ERβ. These results will enable the generation of vasculoprotective estrogen mimetics without classical uterotrophic side-effects.

A first aspect of the invention provides a vasculoprotective composition comprising an ERβ ligand, preferably an agonist, although the compound may be Erβ antagonist.

In particular, post menopausal women suffer from an increased risk of vascular disease and heart disease and are therefore a target population for treatment with agonists of ERβ.

This was unexpected because it was possible that the vasculoprotective effect operates via the recently-discovered ERβ or by another hitherto unknown ER subtype. Another possibility was that the desired vasculoprotective effect is obtained via modification of the signalling to the response elements of "vasculoprotective" genes via intermediary transcription factors, such as SRC-1, TIF-2, A1B1, or via ER-interacting proteins, such as RIP140, RIP160, TIF1, etc.

According to another aspect of the invention, there is provided a pharmaceutical composition useful for the treatment of vasculopathies comprising an ERβ agonist.

According to another aspect of the invention, there is provided the use of an ERβ agonist in the treament of vasculopathies.

According to further aspects of the invention there are provided methods of treating, or preventing, a vasculopathy comprising treating a subject with an ERβ agonist, preferably an ERβ-selective agonist. The vasculopathy may be a fibroproliferative condition. For example, it may be a fibroproliferative condition such as restenosis, angioplasty, chronic allograft rejection, diabetic angiopathy, autoimmune angiopathy, atreriosclerosis, and atherosclerosis.

According to another aspect of the invention there is provided a method of inducing a vasculoprotective effect in a subject, the method comprising contacting the subject with an ERβ agonist.

Alternatively, the vasculoprotective effect may be induced in cells, tissues such as blood vessels or organs. The cells tissues on which may have been produced *in vitro*, may than be placed in a subject. For example in a vasculoprotective effect may be induced and *in vitro* generated or seeded vascular grafts prior to implantation in humans. The gene may be inserted by a virus.

The vasculoprotective effect may be, for example, reducing intimal thickness.

Preferably, the ERβ agonist is selective for ERβ. For example, it may have a binding affinity for ERβ which is at least 10 times, preferably at least 20 times, greater than for ERα.

According to a further aspect of the invention there is provided a method of producing artificial tissues or organs the method including the step of treating the tissue or organ with an ERβ agonist. For example it is known to produce blood vessels by seeding cells, usually from the patient to receive the blood vessel about a former tube and growing those cells. The invention embraces articificial tissues or organs obtainable by such a method.

Methods and compositions in accordance with the invention will now be described, by way of example only, with reference to the accompanying drawings Figs. 1 to 5, in which;

Fig. 1 shows the results of *in situ* expression of ERα and ERβ at 15 min and 7 days after injury. Sense controls (not shown) were negative;

Fig. 2 shows the results of ERα and ERβ expression at different time points, as quantitated as number of grains/400 μm. separately for media and neointima;

Fig. 3 shows dose response plots of 17α estradiol and genistein on a 7 day denudation injury, as quantiated as number of intima nuclei, and on the uterotrophic effect, as quantitated as the 7 day weight of rat uteruses;

Fig. 4 shows a dose response plot of the effect of estradiol and genistein on the proliferative response of rat vascular smooth muscle cells after serum starvation and PDGF-stimulation *in vitro*; and

Fig. 5 gives details of antibodies used in Examples.

1. Expression and localization of ERα and ERβ after carotid denudation trauma in male rats

Expression and localization of ERα and ERβ was investigated by *in situ* hybridisation from paraformaldehyde-fixed parrafin embedded specimens with sense controls.

Carotid denudations were made to Wistar rats purchased from the Laboratory Animal Center, University of Helsinki, Finland. The rats were anaesthetized with 240 mg/kg chloral hydrate i.p.. Buprenorphine (Temgesic, Reckitt Coleman, Hull, England) was given for peri- and postoperative pain relief. Male and ovariectomized female rats weighing 300-400g were used for all experiments.

A transverse incision of the neck was performed. A full exposure of the carotid system was made by cleaving of the ventral edge of the left sternomastoid muscle and omohyoid muscles. The proximal and distal control of the carotid artery was obtained with a 11 mm micro vascular clip. A 2-French Fogarty balloon catheter (Baxter Healthcare Corp, Santa Ana, CA) was introduced into the common carotid artery through the left external carotid artery and inflated with 0.2 ml air. To produce adequate vessel injury the catheter was passed 3 times, balloon inflated, through the common carotid artery. The external carotid artery was ligated after removal of the catheter and the wound was closed.

Evaluation of histological changes was made from midcarotid sections at 0, 15 min, 3 days, 7 days, 14 days and 30 days after denudation injury. The carotids were removed "en block" and fixed in paraformaldehyde.

Histological specimens were fixed in 3% paraphormaldehyde solution for 4 hours, transferred to saline and embedded in paraffin. The number of cell nuclei in the adventia,

media and intima was quantitated from paraffin sections stained with Meyer's hematoxylin-eosin using 400x magnification.

For *in situ* hybridization, the left carotid of male rats was denuded of endothelium and the rats were sacrified at 15 min, 3 days, 7 days, 14 days and 28 days after injury, with a minimum 3 of rats per time point. The specimens of different time points and the non-denuded control specimen were placed on a single organosilane-treated microscopy slide and *in situ* hybridization was performed as described below.

2. *In situ* hybridization

After deparaffinization and rehydration, sections were denatured in 0.2 M HCl, heat-denatured in 2x Saline-Socium Citrate (SSC) at 70°C and treated with proteinase K (1 μg/ml). Sections were then post-fixed with 4% paraformaldehyde, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated and air-dried. Slides were hybridized with antisense or sense RNA probes (described below) overnight at 60°C, washed in 4xSSC, treated with RNAse A (20 μg/ml) and washed sequentially in SSC solutions (2xSSC, 1xSSC, 0.5xSSC, all at room temperature; 0.1xSCC at 50°C; 0.1xSCC at RT) with 1mM DTT. Finally, the slides were rinsed in 0.1xSSC (with 1 mM DTT), dehydrated in graded ethanols and air-dried. Slides were dipped into autography emulsion (NBT 3, Kodak), exposed for 7-14 days, developed, counterstained, dehydrated and mounted with Permount.

3. Probe preparation

The complementary RNA probes were synthesized according to manufacturer's directions (Promega, Madison, WI) in the presence of ^{35}S-UTP (Amersham, International, Willshire, UK) using following cDNA fragments as templates. For ERβ ; a 400 bp *EcoRI-AccI* fragment (from the 5'UTR region) of the rat ERβ cDNA subcloned in a pBluescript KS vector was linearized with *EcoRI* or *AccI* enzymes for the production of antisense and sense transcripts, respectively. For ERα, a 200 bp *BstxI-EcoRI* fragment (from the 3' UTR, F-domain region) of the rat ERα cDNA subcloned to a Bluescript vector was linearized with *SacI* or *EcoRI* enzymes prior to synthesis of antisense and sense probes, respectively. RNA probes transcripted from opposite strands of the same plasmid template, yielding

antisense and sense probes, were adjusted to the same specific radioactivity (minimum 10 000 cpm/μl).

Control non-denuded carotids were compared to denuded carotids removed at 15 min, and 3, 7, 14, and 30 days post denudation. To ensure that the expression levels between different specimens were comparable, all tissue specimens were placed on one slide and hybridized in identical conditions either for ERα or β.

ERα and β mRNAs were expressed constitutively at low level in the vascular tunica media in normal non-denuded cartotids. The level of expression of ERα mRNA remained unaltered throughout the experiment as can be seen from Figs. 1 and 2.

Figure 1 shows the *in situ* expression of ERα and ERβ in rat carotid seven days past denudation. Antisense RNA probes were used with the Lumen (L) facing up. Compared to specimens obtained 15 minutes post denudation, ERβ expression is strongly enhanced in the media (MED) and particularly in the vascular intima (INT) whereas the level of ERα expression was not elevated.

Figure 2 shows the time course of the events. Male rats were denuded as previously described, and the animals were sacrificed at the same points, i.e 15 minutes, 3, 7, and 14 days post injury. Three-fold up-regulation of ERβ mRNA was observed three days after denudation in the media and the level of expression in the media increased to 8-fold on day 7, whereafter it declined. Even more prominent changes in the expression levels were observed in the hyperplastic intima/neointima. Whereas the ERα expression in the intima remained at the level observed in the control vessel media, or at most doubled, the level of ERβ expression in the intima increased nearly 40-fold on day 7 (Fig. 2), whereafter it declined but remained elevated even after 14 days post injury.

4. Dose responses to 17β-E2 and genistein on post denudation carotid trauma and on uterine weight in female rats.

Female adult rats were ovariectomized on day -7 and carotid denudation was performed on day 0 and the animals were killed on day 7 (at the end of the experiment also the uterus was removed, weighed and histology was performed).

Female rats were ovariectomized, placed on a soy-bean free diet (Special Diet Services, UK) (to eliminate effects of phytoestrogens from the diet) for 7 days and both carotids were denuded. One group of animals received 17β-estradiol (17β-E2) (Sigma, St Louis, MO) and the other group genistein (kindly donated by Dr. William Helferich, Michigan State University, or purchased from Plantech, UK) at reducing doses from 2.5 mg/kg/d s.c. downwards, whilst the third group received vehicle only and served as control. 17β–E2 and genistein were dissolved in dimethylsulphoxide (Sigma). Animals were weighed daily, and both drugs were administered subcutaneously (s.c.) using the following doses: 2.5, 0.25, 0.025 and 0.0025 mg/kg in one s.c. injection per day. The animals were killed at 7 days post injury, the uterus and both carotids were removed, the uterus was weighed and both organs were processed for histology as previously described.

Ten denuded carotids of rats receiving only vehicle (DMSO 200 ml/kg/day) were compared to denuded carotids of rats receiving 17-β estradiol and to carotids of rats receiving genistein at escalating doses of 0.0025, 0.025, 0.25 and 2.5 mg/kg/d, three to five carotids at each dose level.

Both 17β–E2 and genistein had a dose-dependent effect on nuclear number in intima, but no measurable effect on the number of nuclei in the media (not shown). (Fig. 3). The line plots indicate that genistein might have been slightly more efficacious (r^2 0.838 vs 0.746) in its vasculoprotective effect.

On the other hand, in the dose range employed, only 17-β estradiol displayed a dose-dependent stimulatory effect on uterine weight (r^2 0.954) while genistein had no effect (r^2 0.96) (Figure 3).

5. Effect on estradiol vs genistein on SMC replication *in vivo*

An aqueous solution of 5-bromo-2'-deoxyuridine and 5-fluoro-2'deoxyuridine (Zymed Laboratories, Inc, San Francisco,CA) was used for labelling of proliferating cells after denudation. For "pulse labelling" a dose of 400 µl of labelling suspension was injected i.v. according to manufacturer's instructions, and the rats were killed exactly 3 h after the pulse. The carotids were fixed as described above and processed for paraffin embedding. BrdU stainings of cross sections were performed using a mouse primary antibody (Bu20a, Dako, A/S, Denmark) and Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Sections were deparaffinized and microwave-treated at 500 W for 2 x 5 min in 0.1 M citrate buffer, pH 6, followed by treatment in 95% formamide in 0.15 M tri-sodium citrate at 70°C for 45 min. Antibody dilutions were made according to manufacturer's instructions. Sections were counterstained with Mayers' haematoxylin and eosin, and the number of positive cells was counted separately from the intimal, medial and adventitial layers.

Both of these ligands also reduced, dose dependently, the replication rate in the intima, as quantitated by the number of BrdU incorporating cells after pulse labelling of the rat.

Table 1 shows the effect of E2 and genistein on the number of proliferating (BrdU-incorporating) cells in the vascular intima seven days after denudation injury.#

Drug dose (mg/kg/d)	No of BrdU incorporating cells	
	17β estradiol	genistein
Nil	36.7 + 7.5	
0.0025	27.3 + 12.5	32.3 + 12.
0.025	6.0 + 3.8	6.0 + 3.8
2.5	10.5 + 2.9	12.3 + 3.7

Animals received BrdU pulse 3 hours before sacrifice.

6. Dose-responses to 17β-E2 and genistein on vascular smooth muscle cell proliferation *in vitro*.

As the results regarding the *in vivo* responses of genistein vs 17β-E2 to vascular trauma suggested a marginally better efficacy of genistein, this possibility was investigated further *in vitro* in the proliferation assays of vascular smooth muscle cells. Rat thoracic aorta smooth muscle cells at 10-12 passage were plated in 96 well tissue culture plates on day -2, left to attach phenyl-red free RPMI 1640 and deprived from serum for additional two days. On day 0, the cells were stimulated by 20 ng/ml Platelet-Derived Growth Factor-B (PDGF-B) (Sigma), or left non-stimulated. Genistein and 17-β estradiol were added to the cultures at the indicated concentrations on day -1, and all cultures were harvested on day 1 after a 24 hour ^{3}H-thymidine (^{3}H-TdR) pulse on day 1.

As seen in Figure 4, both E2 and genistein displayed a dose-dependent anti-proliferative effect on baby rat smooth muscle cells in culture.

7. Expression and localization of ERα AND ERβ in primates after carotid denudation trauma

Because species differences may exist in gene expression, we wanted to confirm the rat results described above in subhuman primates and also to extend the observations to other types of vasculopathies, particularly to allograft fibroproliferative vascular disease in a rat cardiac transplant model and to human cardiac transplantation.

Baboons are a particularly useful animal model as they have 98% sequence identity with the human genome.

Carotid/iliac denudations were performed to baboons Balloon catheter denudation of carotid arteries was performed in 8 male baboons (Papio ursinus) weighing 16-18 kg. Additional 4 baboons served as non-operated controls.The animals were purchased from the Animal Laboratory of the Medical Faculty, University of Stellenbosch, South Africa. The baboons were sedated with ketamine hydrochloride (5mg/kg IM) and anesthesia was

induced with thiopental sodium(5mg/kg IV) and maintained with inhaled halothane. Prophylactic cefazolin sodium (25mg/kg IM,Eli Lilly) was administered and the left common carotid artery was explored by vertical neck incision following the anterior border of sternocleidomastoid muscle. The carotid system was exposed in the carotid triangle and proximal and distal control of the common carotid artery was obtained with small vascular clamps, just proximal to its bifurcation. No heparin was administered. Through a small arteriotomy, a 4-Fr Fogarty balloon catheter (Baxter Healthcare Corp, Santa Ana , CA) was introduced into the distal common carotid artery. It was passed retrograde into the aortic arch and inflated with 1.7 ml of air resulting in a 1.5 lbs pull force and a balloon size of 9 mm when inflated. The inflated balloon was then retrieved under tension while rotating the shaft of the catheter to produce uniform injury. This was repeated three times to ensure sufficient arterial denudation. All procedures were done by the same individual. The catheter was then removed; the arteriotomy closed with interrupted 7-0 monofilament polypropylene sutures, and flow restored. The wound was then closed in layers and buprenorphine (Temgesic, Reckitt, Coleman, Hull, England) 0.25mg/kg IV was given as required, for postoperative pain relief. One baboon was sacrificed at each time point by administering a overdose of pentobarbitol (100 mg/kg IV) and IV potassium chloride. Three minutes before extermination, standard heparin (300U/kg IV) was given. Both carotid arteries were then removed and evaluation of histological changes was madefrom midcarotid sections at 0,15 min and at 2,3,4,7,14 and 28 days post injury.

Pieces of rat uterus were obtained for ER-α specificity controls.

All animals received humane care in compliance with guidelines set forth by the National Institutes of Health, publication No.86-23,Guide for the Care and Use of Laboratory Animals, and the project was approved by the Ethical Committee of the Faculty of Medicine of the University of Stellenbosch, South Africa.

Immunohistochemistry was made from paraffin cross sections using a mouse or rabbit primary antibody and Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Sections were deparaffinized and microwave-treated at 500 W for 2 x 5 min in 0.1 M citrate buffer, pH 6, followed by treatment in 95% formamide in 0.15 M tri-sodium citrate

at +70°C for 45 min. Antibody dilutions were made according to manufacturers instructions. Sections were counterstained with Mayers haematoxylin and eosin, and the total number of positive cells was counted separately from the intimal, medial and adventitial layers using 400x magnification. At least 5 sections were investigated separately of each carotid and the specimen with median intensity of intimal changes was counted.

In the baboon carotid/iliac denudation model 4 baboons were sacrificed w/o injury and at least 1 baboon was sacrificed at 15 min, 2, 3, 4, 7, 14 and 28 d time points post injury. ERβ was found as exclusive receptor in baboon arteries and ERα in baboon uterus. After injury, the intensity of immunohistochemical staining with all three commercial antibodies for ERβ increased considerably and staining colocalized with the vascular SMC. There was no staining with ERα antibodies though the control uterus stained strongly.

ERβ was the only ER located in the baboon ateries found by immunohistochemistry. Specifically tissue samples were contacted with commercially-available ERβ or ERα-selective antibodies.

The staining colocalized with vascular SMC.

8. Analysis of ERα and ERβ expression in rat and human allograft vessels.

In rat and human heart allograft vessels ERβ is the exclusive receptor and ERα in uterus. During acute rejection, the ERβ was shown to be strongly upregulated (same panel of antibodies) and ERα remain non-existent.

Human specimens were obtained from routine enbdomyocardial biopsies performed for diagnostic purposes to U of Helsinki Hospital heart transplant receipients. The biopsies represented no rejection, acute rejections of different histological intensities and of chronic rejection, least 10 specimens per group.

Rat heart transplants were made to abdominal vessels as described (Lemstrom, K., Sihvola, R., Bruggeman C., Häyry, P., and Koskinen, P. abolished by DHPG prophylaxis in the rat. Circulation 1997:95:2614-2616).

Specimens for immunohistochemistry were obtained as described above in relation to baboons.

and following antibodies shown in Fig. 5 were employed, with consistent results:

In rat and human heart allograft vessels ERβ was also found to be the exclusive receptor and ERα dominated in uterus. During acute rejection, the staining for ERβ was strongly upregulated (same panel of antibodies) and the ERα reactivity remain non-existent.

Taken together, the expression patterns of ERβ vs. ERα in subhuman primates and in human and rat allograft models entirely agree with the rat carotid results described in the application and demonstrate ERβ as the exclusive estrogen receptor in arterial tissue and vascular SMC making this a ideal target for drug therapies.

Claims

1. A vasculoprotective composition comprising an ERβ ligand.

2. A vasculoprotective composition according to claim 1 wherein the ERβ ligand is an ERβ agonist.

3. A vasculoprotective composition according to claim 1 wherein the ERβ ligand is an ERβ antagonist

4. A vasculoprotective composition according to claim 1 or claim 2 comprising an ERβ-selective agonist.

5. A pharmaceutical composition useful for the treatment of vasculopathies comprising an ERβ agonist.

6. A pharmaceutical composition according to claim 5 comprising an ERβ-selective agonist.

7. A composition according to claim 4 or 6 in which the binding affinity of the ERβ agonist to ERβ is at least 10 times greater than the binding affinity to ERα.

8. A composition according to claim 7 in which the binding affinity of the agonist to ERβ is at least 20 times greater than to ERα.

9. The use of an ERβ agonist in the treatment of vasculopathies.

10. The use of an ERβ-selective agonist in the treatment of vasculopathies.

11. The use according to claim 10 in which the vasculopathy is a fibroproliferative condition.

12. The use according to claim 11 in which the fibroproliferative vasculopathy is selected from restenosis, angioplasty, chronic allograft rejection, diabetic angiopathy, autoimmune angiopathy, arteriosclerosis, and atherosclerosis.

13. A method of inducing a vasculoprotective effect in a subject, the method comprising treating the subject with an ERβ agonist.

14. A method of inducing a vasculoprotective effect according to claim 13 in which the ERβ agonist has a higher affinity for ERβ than ERα.

15. A method of inducing a vasculoprotective effect in a subject according to claim 14 in which the binding affinity of the agonist to ERβ is at least 10 times greater than to ERα.

16. A method of inducing a vasculoprotective effect in a subject according to claim 15 in which the binding affinity of the agonist to ERβ is at least 20 times greater than to ERα.

17. A method of inducing a vasculoprotective effect in which the effect is decrease of intimal thickness.

18. A method according to any one of claims 13 to 17 in which the vasculoprotective effect is induced to treat a fibroproliferative vasculopathy.

19. A method according to claim 18 in which the fibroproliferative vasculopathy is selected from restenosis, angioplasty, chronic allograft rejection, diabetic angiopathy, autoimmune angiopathy, ateriosclerosis and atherosclerosis.

20. A composition, use or method according to any preceding claim in which the ERβ selective agonist is genistein or a chemical derivative or structural analogue thereof.

21. A use or method according to any one of claims 9 to 20 in which uterotrophic effects are minimised or do not result.

22. A method according to any one of claims 13 to 21 in which the subject is a mammal.

23. A method according to claim 22 in which the mammal is a primate.

24. A method according to claim 23 in which the mammal is human.

25. A method according to claim 22, 23 or 24 in which the mammal is female.

26. A method according to claim 25 in which the female is post-menopausal.

27. A method of producing artificial tissues or organs the method including the step of treating the tissue or organ with an ERβ agonist.

28. A method according to claim 27 in which the tissue or organ is a blood vessel.

29. Articificial tissues or organs obtainable by a method according to claim 27 or 28.

ABSTRACT

VASCULOPROTECTOR

The invention relates to a method of inducing a vasculoprotective effect in a subject, the method comprising treating the subject with an ERβ agonist.

FIG.1

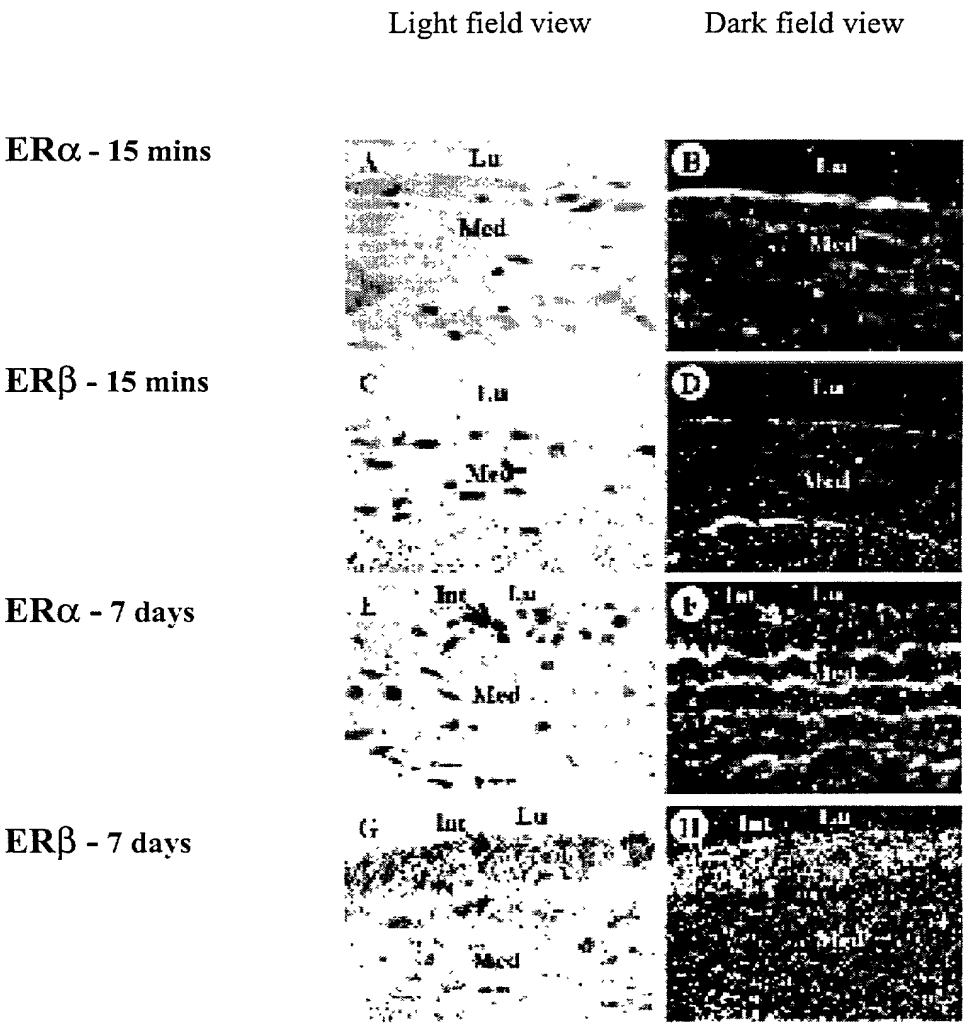
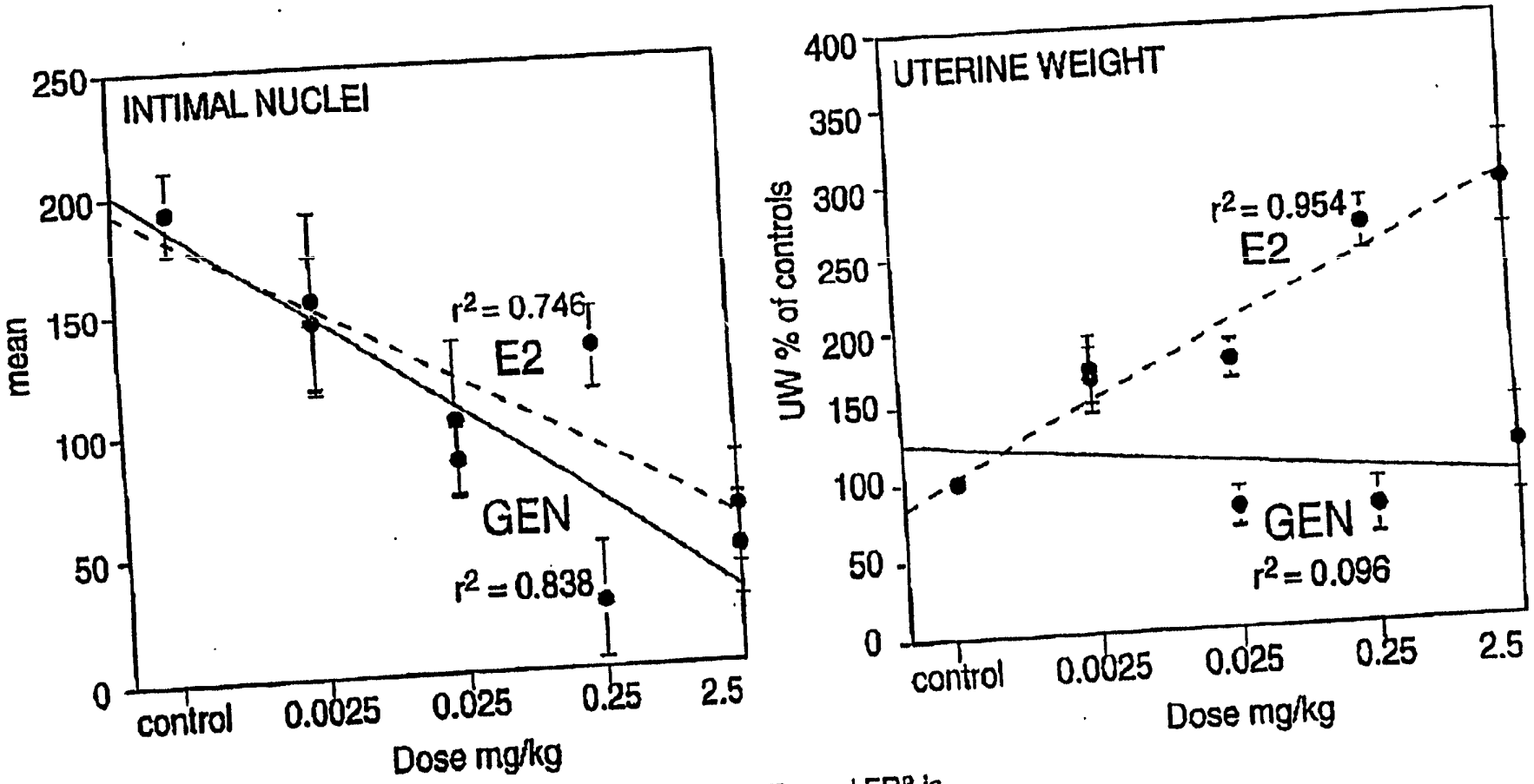


FIG.2



Binding affinity Ki (nM) of 17α estradiol and genistein to ERα and ERβ is, respectively, 0.13 and 0.12 for E2 and 2.6 and 0.3 for GEN.

FIG. 3

FIG. 4

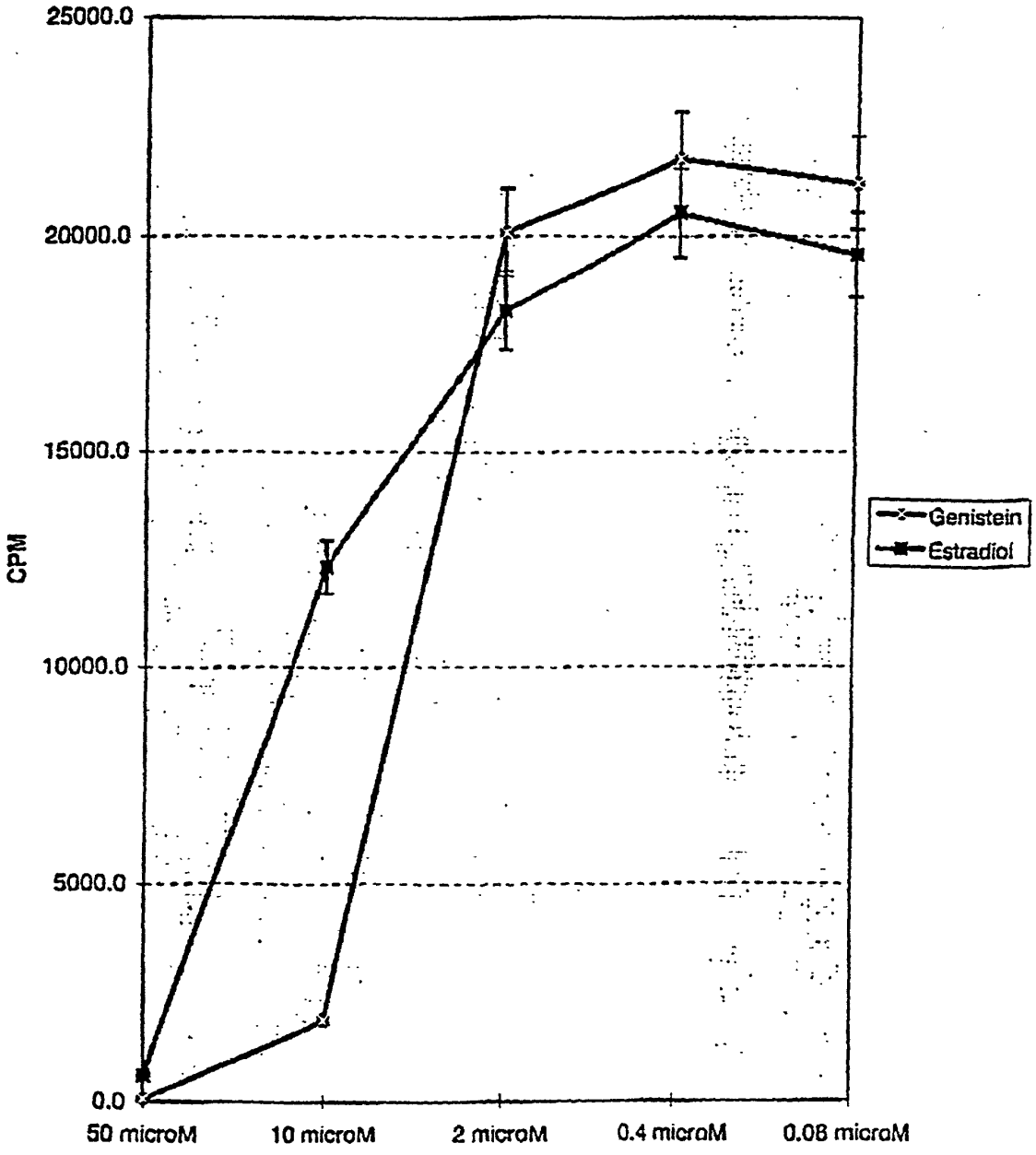


FIG. 5

ESTROGEN ANTIBODIES								
Name	Type	Species reactivity	Specificity/working concentration	Antigen/ recognition sequence	Conc.	Cat. #	Company	References
Estrogen receptor, nuclear	Rabbit	Rat, human	IHC 10ug/ml	Synthetic peptide aa.	200ug of protein A pu-	06-629	Upstate	Enmark, E. L., et al., Proc. Natl. Acad. Sci. USA 93: 5925-5930, 1996.
			IP	54-71 of rat, mouse.	rified IgG in 200ul of		biotechnology	
			WB 1-2ug/ml	Aa. 46-63 of human.	0.1 M Tris-glycine.			Byers, M., et al., Mol. Endo. 11: 172-182, 1997
Estrogen receptor	MoAb	Human, bovi-	IHC 1:50-1:100	Purified, SDS-denatu-	200ug of protein A	05-394	Upstate	Evans, R. M., Science 240: 889-895, 1988.
		ne, rat,	IP	red calf uterus	purified mouse IgG in		biotechnology	
		mouse	WB 0.5-2ug/ml	ER-receptor	400ul of 10mM PBS			Green, S., et al., Nature 324: 615-617, 1986.
Estrogen receptor, nuclear	MoAb	human	IHC 1:40-1:60	Procaryotic recombi-	Lyophilised tissue	NCL-ER-6F1	NovoCastr	Bevitt, D. J., Piggot, N., et al., New monoklonal antibodies to oestrogen and progestrone receptors effective for paraffin section IHC. Journal of Pathology. 183: 228-232, 1997.
		mouse	Flowc.	nant protein corres-	culture supernatant.			
			WB 1:50-1:100	bonding to the full-	/1ml Aqua			Clark, G. M., McGuire, W. L., The clinical usefulness of oestrogen-receptor and other markers of hormone dependence. Proceedings of the Royal Society of Edinburgh. 95B: 145-150, 1989.
				length alpha form of				
				the ER-reseptor mo-				
				lecule.				Henry, J. A., Angus, B., Horne, C. H. W., Oestrogen receptor and oestrogen regulated proteins in human breast cancer: a review. KEIO Journal of Medicine. 38: 241-261, 1989.
								Shintaku, P., Said, J. W., Detection of oestrogen receptors with monoklona antibodies in routinely processed formalin-fixed paraffin sections of breast carcinoma. American Journal of Clinical Pathology. 87: 161-167, 1987.
Estrogen receptor, nuclear	MoAb	human	IHC 1:50-1:75	N-term. domain of the	Recombinant human	M 7047	DAKO	Kumar, V., et al., Functional domains of the human oestrogen receptor.
			WB	reseptor (A/B region)	ER-reseptor protein			Cell 51: 941-51, 1987.
					(tissue culture super-			Sannino, P., Shousha, S., Demonstration of oestrogen receptors in paraffin
					natant /1ml RPMI 1640)			wax sections of breast carcinoma using the monoklonal antibody 1D5 and microwave oven processing. J. Clin. Pathol. 47: 90-2, 1994.
Estrogen receptor beta, cytoplasmic	Rabbit	Rat	IHC 5-10ug/ml	rat peptide	50ug/50ul PBS	PA1-310	ABR	Li, X., Schwartz, P. E., Rissman, E. F., Distribution of oestrogen receptor-beta-like immunoreactivity in rat forebrain. Neuroendocrinology 66: 63-67, 1997
		mouse	WB 1ug/ml	COOH-terminal				
				aa. 467-485	Control peptide	PEP-007		
Estrogen receptor beta, cytoplasmic	Rabbit	Rat	IHC 1-2ug/ml	rat peptide	50ug/50ul PBS	PA1-311	ABR	Alves, S. E., et al., Differential colocalization of oestrogen receptor beta with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: An immunocytochemical study. Proc. Natl. Acad. Sci. USA 95(6): 3281-3286, 1998.
		mouse	WB 1-2ug/ml	NH2-terminal				
				aa. 55-70	Control peptide	PEP-011		
Estrogen receptor alpha	MoAb	Human	IHC 5ug/ml	human peptide	50 µg/100 µl PBS	MA1-310	ABR	Traish, A., et al., Development and characterization of monoklonal antibodies to a specific domain of human oestrogen receptor. Steroids 55: 196-208, 1990.
		Rat	IP 5ug/ml	DNA-binding dom.	(pre-diluted Ascites)			
			WB 5 µg/ml	aa. 247-261	Control peptide	PEP-013		
			(1ug/ml)					

KB123US #3

COMBINED DECLARATION AND POWER OF ATTORNEY FOR JOINT INVENTORS

1. As below named joint inventors, we hereby declare that our addresses and citizenship are as stated below next to our names. We believe we are the original and first inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

VASCULOPROTECTOR

the specification of which:

[ ] is attached or

[X] was filed on December 13, 2000 as Serial No. 09/719,658.

2. We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

3. We acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. 1.56.

4. [X] We hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by us on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application Serial No	Date of Filing (day, mo., yr.)	Priority Claimed under 35 U.S.C. § 119	
Great Britain	9814620.2	6 July 1998	[X] Yes	[ ] No
			[ ] Yes	[ ] No
			[ ] Yes	[ ] No

5. [X] We hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), § 365(c) of any PCT international application designating the United States of America, and § 119(e) of any United States provisional application(s) that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application(s) and the filing date of this application:

Application Serial No.	Filing Date	Status
PCT/GB99/02157	6 July 1999	Pending

6. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

7. As named inventors, we hereby appoint the following attorneys of Wiggin & Dana to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Dale L. Carlson, Reg. No. 28,784; Todd E. Garabedian, Ph.D., Reg. No. 39,197; Gregory S. Rosenblatt, Reg. No. 32,489; William A. Simons, Reg. No. 27,096; William B. Slate, Reg. No. 37,238, and Jody L. DeStefanis, Reg. No. 44,653.

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9. [X] As named inventors, we hereby appoint the attorneys listed in paragraph 7 as our domestic representatives for the invention identified in paragraph 1 with full power of substitution and revocation, to transact all business in the U.S. Patent and Trademark Office and in the U.S. courts in connection therewith. They also designated as domestic representative on whom process or notice of proceedings affecting the application or patents issuing therefrom may be served.

[X] We hereby authorize the U.S. attorneys named in paragraph 7 to accept and follow instruction from Withers & Rogers as to any actions to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and us. In the event of a change in the persons from whom instructions may be taken, we will notify the U.S. attorneys.

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